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1. **Your reference** — 444.78730/000

2. **Patent application number** *(The Patent Office will fill in this part)* — 0225551.1 — 01 NOV 2002

3. **Full name, address and postcode of the or of each applicant** *(underline all surnames)*

Bioparken AS
Fredrik A Dahls vei 20
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0849817 20d

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give country/state of incorporation — Norway

4. **Title of the invention** — ASSAY METHOD

5. **Name of your agent** *(if you have one)* — Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

179 Queen Victoria Street
London
EC4V 4EL

Patents ADP number *(if you know it)* — 166001

6. **If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and** *(if you know it)* **the or each application number**

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. **If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application**

Number of earlier application	Date of filing *(day / month / year)*

8. **Is a statement of inventorship and of right to grant of a patent required in support of this request?** *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
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Yes

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Request for preliminary examination and search *(Patents Form 9/77)*	-
Request for substantive examination *(Patents Form 10/77)*	-
Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application.

Signature Frank B Dehn & Co Date 1st November 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Julian Cockbain
020 7206 0600

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Assay Method

This invention relates to an assay method for detecting fungal infection of fields and vegetables, and to compounds and kits for use in such assays.

Almost one third of the carrot crop is lost worldwide due to pests and diseases.

While chemical treatment of carrot growing fields and of harvested carrots can be used to reduce the loss in the carrot crop, this is expensive and means that the carrots can not be sold as "organic".

There is thus a pressing need for a diagnostic method with the use of which loss in crop yield may be reduced.

Root vegetables like carrots are particularly susceptible to pathogens present in the soil in which they are grown and especially to fungal infection. Such fungal infection can cause damage to the carrots while still in the ground or the damage may occur later during post-harvest storage.

One especially damaging fungal infection of carrots is called liquorice rot and is caused by the fungus Mycocentrospora acerina. Another especially damaging fungal infection of carrots is called crater rot and is caused by the fungus Fibularhizoctonia carotae. Both infections develop during post-harvest storage and render the carrots essentially worthless.

Fungal infection of carrot growing fields may, as mentioned above, be treated by spraying the fields with antifungal agents, e.g. metalaksyl. Alternatively the infected fields may be used for other crops not sensitive to fungal infection by M. acerina or F. carotae until the infection has disappeared. However waiting for the infection to clear is a long and uncertain business as the fungus may have other host species available and as viable fungal spores can remain

dormant in the soil for years.

We have now found that soil from fields in which carrots might be grown may be analysed to determine whether the fields are infected with M. acerina or F. carotae thus enabling the grower to decide whether to spray with an antifungal agent or to avoid planting such fields with carrots until a later season when the infection has disappeared. Likewise tissue or surface soil from symptom-free carrots may be tested, e.g. before or shortly after harvesting, to determine whether treatment with a fungicide to prolong storage life is necessary or to determine whether the carrots should be used (e.g. sold, cooked, bottled, canned etc) promptly rather than stored for prolonged periods.

Thus viewed from one aspect the invention provides an assay method for detecting fungal infection of soil or vegetables by pathogenic fungal species, said method comprising:

obtaining a sample of soil or vegetable; treating said sample to lyse fungal cells therein; using an oligonucleotide primer pair, effecting a polymerase chain reaction on DNA released by lysis of the fungal cells; and detecting DNA fragments generated by said polymerase chain reaction;

wherein said primer pair comprises an 18- to 24-mer having the ability to hybridize to one of the oligonucleotide sequences of formulae Ia, Ib, IIa, IIb, IIIa, IIIb, IVa and IVb:

5' - GTT TGA ATG GAG TCC GAC CG - 3' (Ia)
5' - CGG CGT ACT TGC TTC GGA GC - 3' (Ib)
5' - TGG GAT TAA CGG GCA GAG AC - 3' (IIa)
5' - TTT CGC ATT CGG AGG CTT GG - 3' (IIb)
5' - CGG TCG GAC TCC ATT CAA AC - 3' (IIIa)
5' - GCT CCG AAG CAA GTA CGC CG - 3' (IIIb)
5' - GTC TCT GCC CGT TAA TCC CA - 3' (IVa)
5' - CCA AGC CTC CGA ATG CGA AA - 3' (IVb).

In the assay method of the invention, the primer pair preferably comprises an 18- to 24-mer having the ability to hybridize to one of the oligonucleotide sequences of formulae Ia, Ib, IIa or IIb. Even more preferably the primer pair comprises a pair of 18- to 24-mers having the ability to hybridize to a pair of the oligonucleotide sequences of formulae Ia and Ib or IIa and IIb. Less preferably the second primer may be a general primer that binds to all or substantially all fungal DNA. Such general primers, typically also 18- to 24-mers are known and will still allow the polymerase chain reaction to function efficiently. Indeed such general primers are known which hybridize to DNA of all fungi, all oomycetes and all plants.

Examples of such general primers include:

5' - TCC GTA GGT GAA CCT GCG G - 3'	(A)
5' - GCT GCG TTC TTC ATC GAT GC - 3'	(B)
5' - GCA TCG ATG AAG AAC GCA GC - 3'	(C)
5' - TCC TCC GCT TAT TGA TAT GC - 3'	(D)
5' - GGA AGT AAA AGT CGT AAC AAG G - 3'	(E)

General primers (A) and (E) are especially useful for use with specific primers which hybridize to sequences of formulae IIIa, IVa, Ib and IIb. General primer (D) is especially useful for use with primers that hybridize to sequences of formulae IIIb, IVb, Ia and IIa. General primer (B) is especially useful for use with primers that hybridize to sequences of formula Ia and IIa. General primer (C) is especially useful for use with primers that hybridize with sequences of formula Ib and IIb.

By "having the ability to hybridize to" is meant having the ability to anneal to DNA incorporating such a sequence at the site of that sequence under conditions under which primer annealing in the performance of a PCR reaction may be effected.

One primer is preferably a compound consisting of or comprising a sequence of formula Ia, Ib, IIa, IIb, IIIa, IIIb, IVa or IVb or a derivative thereof in which up to 5 nucleotide residues: are omitted or replaced by different residues; or are inserted; or are omitted from or added to the 3' or 5' termini. In the case of such derivatives, preferably no more than one residue is omitted at a 3' terminus and no more than 3 at a 5' terminus, preferably no C residue is replaced by an A residue, preferably no more than 3 C or G residues are replaced, preferably no more than one omission or insertion within the listed sequence occurs and preferably any extension at the 3' termini is 5'-GCGGG-3', 5'-GCAGC-3', 5'-GTGCA-3', 5'-ATTGT-3', 5'-CCTTT-3', 5'-GCTGC-3', 5'-ACCCA-3' or 5'-CAAAT-3' or a fragment from the 5' end thereof for Ia, Ib, IIa, IIb, IIIa, IIIb, IVa and IVb respectively. More preferably, in such derivatives, no more than 3 residues are replaced or omitted, and particularly no more than 2 C or G residues are replaced.

Preferably one of the primers is a compound consisting of or comprising a sequence of formula IIIa, IIIb, IVa, or IVb or a such derivative thereof. More preferably the primer pair comprises two compounds consisting of or comprising sequences of formula IIIa and IIIb or IVa and IVb, or such derivatives thereof.

In alternative preferred derivatives, none of the C or G residues are replaced or omitted. In further preferred derivatives any replacement, omission or addition of nucleotides is made in the 5' portion of the primer sequence, e.g. in the 5' half of the primer sequence. Preferably 8 or more nucleotide residues, e.g. 8, 9 or 10 residues, at the 3' end of the primers are not altered. Fragments of such derivatives which have the ability to hybridise to sequences of formula Ia, Ib, IIa, IIb, IIIa, IIIb, IVa or IVb are also included.

"Substantially homologous" as used herein in connection with a nucleic acid sequence includes those sequences having a sequence homology or identity of approximately 60% or more, e.g. 70%, 75%, 80%, 85%, 90%, 95%, 98% or more, with a particular sequence and also functionally equivalent variants and related sequences modified by single or multiple base substitution, addition and/or deletion. By "functionally equivalent" in this sense is meant nucleotide sequences which have the ability to hybridise to sequences of formula Ia, Ib, IIa, IIb, IIIa, IIIb, IVa or IVb in accordance with the definition above. Such functionally equivalent variants may include synthetic or modified nucleotide residues providing the hybridisation function of the primer is retained.

Sequences which "hybridise" as used herein in connection with the definition of derivative primers are those sequences which bind (hybridise) to a particular DNA sequence under conditions of low or preferably high stringency. Such conditions are well known and documented in the art. For example such sequences may hybridise to a particular DNA sequence under non-stringent conditions (e.g. 6 x SSC, 50% formamide at room temperature) and can be washed under conditions of low stringency (e.g. 2 x SSC, room temperature, more preferably 2 x SSC, 42 C) or conditions of higher stringency (e.g. 2 x SSC, 65 C) (where SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2).

Generally speaking, sequences which hybridise under conditions of high stringency are included within the scope of the invention.

The 18 to 24-mer primers may be prepared by conventional chemical techniques, e.g. solid state synthesis.

It is especially preferred that two primer pairs be used in the method of the invention, one pair comprising primers hybridizing to sequences of formulae Ia and/or

Ib (or less preferably IIIa and/or IIIb) and another comprising primers hybridizing to sequences of formulae IIa and/or IIb (or less preferably IVa and/or IVb), i.e. respectively to detect M. acerina and F. carotae infection.

Such use of two primer pairs may be simultaneous or, more preferably in separate PCR reactions on aliquots of the sample.

The primers are themselves novel compounds and form a further aspect of the invention.

Viewed from this aspect the invention provides an 18- to 24-mer oligonucleotide primer hybridizable to an oligonucleotide sequence selected from those of formulae Ia, Ib, IIa, IIb, IIIa, IIIb, IVa and IVb.

Viewed from a still further aspect the invention provides a primer composition comprising a pair of 18- to 24-mer oligonucleotide primers at least one of which is hybridizable to an oligonucleotide sequence of formula Ia, Ib, IIa, IIb, IIIa, IIIb, IVa or IVb, optionally together with a carrier.

The composition of the invention preferably comprises a pair of 18- to 24-mer oligonucleotide primers hybridizable to the oligonucleotide sequences of formulae Ia and Ib and/or a pair of 18- to 24-mer oligonucleotide primers hybridizable to the oligonucleotide sequences of formulae IIa and IIb.

For the detection phase of the method of the invention, it is possible to use labelled primers, e.g. radiolabelled or labelled with a chromophore or fluorophore or an enzyme. Such labelled versions of the primers of the invention and compositions containing them form further aspects of the invention.

Viewed from a yet still further aspect the invention provides a kit for the performance of the assay method of the invention, said kit comprising at least one primer pair according to the invention together with instructions for the performance of the

assay method. Advantageously the kit also comprises a DNA-polymerase, e.g. Taq-polymerase, and especially advantageously the kit includes a set of components (e.g. chemical compositions) for DNA extraction.

The soil sample, approximately 0.5g for each PCR reaction, is preferably taken from a larger sample, for example at least 100g, more preferably at least 200g, e.g. up to 1000g, which has been mixed (e.g. by physical intermingling of the larger example or by addition together of aliquots of different parts of the larger sample) so that the sample analysed is representative of the larger sample - this is in distinct contrast to conventional PCR-based DNA analysis of soil where such representative sampling is not effected. The sample may be taken from a single location or it may be the combination of samples from multiple locations in a growing area (e.g. a field). The separate analysis of multiple samples from different locations in a field is preferable but, for reasons of economy, analysis of a composite sample may be preferred.

The soil is preferably taken at a depth of up to 30 cm, especially 1 to 20 cm. Samples are also preferably taken from both the margins and the central section of the growing area, preferably at a distance of at least 3m from the edge of the growing area (e.g. from a hedge, ditch, fence, track, etc).

Where the field is already in use in vegetable, e.g. carrot, production, the soil samples are advantageously taken from the soil within 10 cm, more preferably within 5 cm of the growing vegetables. Particularly conveniently, vegetables are uprooted and the soil on the uprooted vegetables is used for the assay.

We have found that humus in the soil reduced the accuracy of the assay method of the invention and thus pathogen DNA extraction from the soil samples preferably involves the following steps:

1) contact a sample of about 0.1 to 1g, preferably about 0.5g, soil taken from a mixed sample of at least 100g, preferably at least 200g, soil with a fungal cell lysing agent;
2) centrifuge at least 10000xg for at least 10 minutes and collect the supernatant;
3) contact the supernatant with a particulate DNA-binding agent;
4) centrifuge and collect the DNA-bearing particulate;
5) suspend the particulate in an aqueous solution of a chaotropic agent (e.g. aqueous guanidine thiocyanate solution), centrifuge and collect the DNA-bearing particulate;
6) repeat step (5) at least once;
7) suspend the particulate in aqueous salt ethanol wash solution, centrifuge and collect the DNA-bearing particulate;
8) repeat step (7) at least once;
9) suspend the particulate in an aqueous solution of a DNA-release agent;
10) centrifuge and collect the DNA-containing supernatant; and optionally
11) resuspend the particulate in an aqueous solution of a DNA-release agent, centrifuge and collect and combine the supernatant.

As compared with DNA-from-soil extraction using the commercially available kit FastDNA SPIN Kit for Soil (available from Qbiogene Inc/Bio 101 of Carlsbad, California, USA), this DNA extraction procedure involves a significantly longer post-lysis centrifugation, and repeated rinsing of the DNA-bearing particulate. In general also significantly larger volumes of release agent to free the DNA from the binding matrix should be used. Nonetheless the resultant procedure is one which provides good results for the full range of soil types in which vegetables are grown. The prior art extraction

techniques in comparison are very sensitive to the soil type under investigation.

Thus viewed from a further aspect the invention provides a process for the extraction of nucleic acid (e.g. DNA) from soil which process comprises:

1) contact a sample of about 0.1 to 1g, preferably about 0.5g, soil taken from a mixed sample of at least 100g, preferably at least 200g, soil with a fungal cell lysing agent (e.g. a ceramic and silica particulate);
2) centrifuge at least 10000xg for at least 10 minutes and collect the supernatant;
3) contact the supernatant with a particulate DNA-binding agent;
4) centrifuge and collect the DNA-bearing particulate;
5) suspend the particulate in an aqueous solution of a chaotropic agent (e.g. an aqueous guanidine thiocyanate solution), centrifuge and collect the DNA-bearing particulate;
6) repeat step (5) at least once;
7) suspend the particulate in aqueous salt/ethanol wash solution (generally with a water/ethanol volume ratio of about 1:10), centrifuge and collect the DNA-bearing particulate;
8) repeat step (7) at least once;
9) suspend the particulate in an aqueous solution of a DNA-release agent;
10) centrifuge and collect the DNA-containing supernatant; and optionally
11) resuspend the particulate in an aqueous solution of a DNA-release agent (e.g. DNase in pyrogen-free water), centrifuge and collect and combine the supernatant.

Viewed from a further aspect the invention provides a kit for nucleic acid (e.g. DNA) extraction from soil,

which kit comprises:

i) a fungal cell lysing agent;
ii) a DNA-binding particulate;
iii) an aqueous solution of a chaotropic agent (e.g. guanidine thiocyanate);
iv) an aqueous solution of salt and ethanol; and
v) an aqueous solution of a DNA-release agent;

together with instructions for the use of said kit in the process of the invention.

Where the sample under analysis is of vegetable tissue rather than soil, it is preferably surface tissue, in particular root (or tuber) surface tissue. Such a sample may be taken for example by peeling the root (or tuber surface) optionally after washing, wiping or rinsing to remove soil. Such samples may be taken at any stage during growth or storage but will preferably be taken from 1 week before harvesting to 3 months post harvesting, more particularly from harvesting to 2 months post harvesting. Where, as is preferred, the vegetable is carrot, we have found that unsaturated organic compounds in the carrot root reduced the accuracy of the assay method of the invention and thus pathogen DNA extraction from vegetable tissue samples preferably involves the following steps:

i) contact at least 20 mg of dry powdered plant tissue (preferably surface tissue such as peel) with at least 5 μL/mg dry tissue of an aqueous fungal cell lysing agent;
ii) incubate;
iii) mix with at least 4.5 μL/mg dry tissue of an aqueous solution of a protein and polysaccharide precipitating agent;
iv) centrifuge and collect DNA-containing supernatant;
v) filter;

vi) contact DNA-containing filtrate with a DNA-binding substrate and centrifuge;

vii) wash the DNA-carrying substrate with an aqueous ethanolic solution, centrifuge and remove the liquid phase;

viii) repeat step (vii) at least once;

ix) dry the DNA-carrying substrate; and

x) contact the substrate with an aqueous solution of a DNA release agent, centrifuge and collect the DNA-containing supernatant.

As compared with DNA-from-plant-tissue extraction using the commercially available GenElute Plant Genomic DNA kit (available from Sigma), this DNA extraction procedure involves the use of dry powdered plant tissue, larger volumes of lysing and precipitation solutions and drying of the DNA-carrying substrate to remove ethanol. Nonetheless the procedure does provide significantly better results and thus viewed from a further aspect the invention provides a process for the extraction of pathogen DNA from host vegetable tissue, which process comprises:

i) contact at least 20 mg of dry powdered plant tissue (preferably surface tissue such as peel) with at least 5 μL/mg dry tissue of an aqueous fungal cell lysing agent;

ii) incubate;

iii) mix with at least 4.5 μL/mg dry tissue of an aqueous solution of a protein and polysaccharide precipitating agent;

iv) centrifuge and collect DNA-containing supernatant;

v) filter;

vi) contact DNA-containing filtrate with a DNA-binding substrate and centrifuge;

vii) wash the DNA-carrying substrate with an aqueous ethanolic solution, centrifuge and remove the

liquid phase;

viii) repeat step (vii) at least once;

ix) dry the DNA-carrying substrate; and

x) contact the substrate with an aqueous solution of a DNA release agent, centrifuge and collect the DNA-containing supernatant.

Viewed from a still further aspect the invention provides a kit for pathogen DNA extraction from host vegetable tissue, which kit comprises:

a) a fungal cell lysing agent;

b) an aqueous solution of a protein and polysaccharide precipitating solution;

c) a DNA-binding substrate;

d) an aqueous ethanolic wash solution; and

e) an aqueous solution of a DNA release agent;

together with instructions for the use of said kit for pathogen DNA extraction from host vegetable tissue.

In these techniques, the fungal cell lysing agent may for example be an enzyme (e.g. L1393 or L1412 from Sigma) or a buffered surfactant (e.g. cetyltrimethylammonium bromide, N-lauroylsarcosine or sodium dodecyl sulphate). Alternatively, mechanical means such as grinding in liquid nitrogen, may be used.

Proteins, polysaccharides and nucleic acids can be separated in these techniques by different strategies. Thus proteins can be precipitated leaving the nucleic acid in solution, for example by adjusting the osmolality of the solution, e.g. by the addition of salts, generally high concentration salt solutions, for example 3M sodium acetate. Proteins can alternatively be extracted using organic solvents such as chloroform or phenol.

DNA extracted from the samples will typically be purified before being subjected to PCR using the primers

of the invention. This can be effected in conventional fashion, e.g. chromatographically. Thus for example Micro Bio-Spin chromatography columns (available from BioRad, Hercules, California, USA) may be used together with insoluble polyvinylpolypyrrolidone powder (e.g. P6755 from Sigma) to purify the DNA.

For the primer pair which hybridizes to the sequences of formula Ia and Ib, the amplified section of DNA is about 294 bp while for the pair which hybridizes to the sequences of formulae IIa and IIb, the amplified section of DNA is about 359 bp.

The PCR reaction itself can again be effected conventionally, e.g. using the primer pair, the four deoxynucleotide triphosphates and a heat stable DNA polymerase (e.g. Taq polymerase, available from Roche). Generally at least 25, more preferably 30 to 50, cycles of the PCR reaction will be sufficient.

The amplified DNA, if present, may then be detected by conventional techniques, e.g. gel separation or hybridization to labelled probes (for example radiolabelled or chromophore/fluorophore labelled probes). Where labelled probes are used, these may typically comprise labelled versions of one of the primer pair or labelled oligonucleotides able to hybridize specifically to the PCR-amplified fragment detected by a photodetector during PCR amplification or taken up by a porous substrate which is then treated with the labelled probe and rinsed, whereafter the signal from the probe retained on the substrate may be detected, e.g. photometrically or using a radiation detector. Where more than one primer pair is used in the PCR reaction, more than one probe will likewise be used and these may be labelled in the same or different fashion, e.g. using labels with different characteristic absorption or emission energies or wavelengths.

The detection of the amplified DNA may be used to provide a qualitative, semi-quantitative or quantitative

indication of the pathogen infestation of the soil sample, e.g. a value in cells per unit weight or an indication that the pathogen content of the soil is above or below a predetermined threshold value, e.g. boundary value for the decision to plant or not plant a particular vegetable crop or the decision to apply or not apply a fungicide.

In a particularly preferred embodiment of the method of the invention, aliquots of the soil sample are also tested in similar fashion for the presence of the fungal pathogens responsible for other vegetable root disease, e.g. cavity spot (caused by Pythium species, especially P. viola and P. sulcatum), ring rot (caused by Phytopthora species, in particular P. megasperma), grey mould (caused by Botrytis cinerea), Sclerotina rot (caused by Sclerotinia sclerotiorum), Chalaropsis rot (caused by Chalaropsis thielaviodes), and other diseases caused by Alternaria dauci, Cercospora carotae and Rhizoctonia solani. Of these, testing for Pythium species in soil samples is the most important as these can cause major crop loss during the growth period. Thus if it is detected the field should be treated with a fungicide (e.g. metalaxyl) or used for a crop other than carrots.

For Pythium the primer pairs conveniently comprise a pair of 18- to 24-mers at least one of which has the ability to hybridize to one of the oligonucleotide sequences of formulae Va to XIVb below. The primer pairs used in this regard are preferably a pair of compounds consisting of or comprising sequences of formulae Va to XIVb below or derivatives thereof in which up to 5 nucleotide residues: are omitted or replaced by different residues; or are inserted; or are omitted from or added to the 3' or 5' termini. In the case of such derivatives, preferably no more than one residue is omitted at a 3' terminus and no more than 3 at a 5' terminus, preferably no C residue is replaced by

an A residue, preferably no more than 3 C or G residues are replaced, preferably no more than one omission or insertion within the listed sequence occurs and preferably any extension at the 3' termini is 5'-GGCGC-3', 5'-GCCGA-3', 5'-GGCTG-3', 5'-AGGCC-3', 5'-GGTCG-3', 5'-CCAAA-3', 5'TTATG-3', 5'-AACAC-3', 5'-TATGC-3' 5'-CAGAT-3', 5'-CAACA-3', 5'-CCACC-3', 5'-TGCTG-3', 5'-ACAGG-3', 5'-CCGGC-3', 5'-TTTGC-3', 5'-AGACA-3', 5'-AGAAG-3', 5'-CGAGA-3', or 5'-GTTTG-3', or a fragment thereof from the 5' end thereof for Va, Vb, VIa, VIb, VIIa, VIIb, VIIIa, VIIIb, IXa, IXb, Xa, Xb, XIa, XIb, XIIa, XIIb, XIIIa, XIIIb, XIVa and XIVb. More preferably, in such derivatives, no more than 3 residues are replaced or omitted, and particularly no more than 2 C or G residues are replaced.

Thus, for Pythium, at least one of the primers Va, Vb, VIa, VIb, VIIa, VIIb, VIIIa, VIIIb, IXa, IXb, Xa, Xb, XIa, XIb, XIIa, XIIb, XIIIa, XIIIb, XIVa and XIVb

5' - TCT TCT TTA CCC CAC AAG TGA - 3'	(Va)
5' - GCC GCT TTA TTG TGG TCT - 3'	(Vb)
5' - ATG TGT GTG TGC GGG ACT - 3'	(VIa)
5' - CCA CTC CCC AAA GAG AGA AGT - 3'	(VIb)
5' - ATG CAG AGG CTG AAC GAA - 3'	(VIIa)
5' - CTG TAT TCA TAG CCG AAA CGA - 3'	(VIIb)
5' - CGC TGT GGT TGG TAT ATT TGT - 3'	(VIIIa)
5' - GCC AAT TGC ACA AGT ACA AA - 3'	(VIIIb)
5' - CAG CGG TTG GTA TAT TCG TT - 3'	(IXa)
5' - AAA AAG AAG TGC ACA AAT AGA TGA - 3'	(IXb)
5' - TCA CTT GTG GGG TAA AGA AGA - 3'	(Xa)
5' - AGA CCA CAA TAA AGC GGC - 3'	(Xb)
5' - AGT CCC GCA CAC ACA CAT - 3'	(XIa)
5' - ACT TCT CTC TTT GGG GAG TGG - 3'	(XIb)
5' - TTC GTT CAG CCT CTG CAT - 3'	(XIIa)
5' - TCG TTT CGG CTA TGA ATA CAG - 3'	(XIIb)
5' - ACA AAT ATA CCA ACC ACA GCG - 3'	(XIIIa)
5' - TTT GTA CTT GTG CAA TTG GC - 3'	(XIIIb)

5' - AAC GAA TAT ACC AAC CGC TG - 3' (XIVa)

5' - TCA TCT ATT TGT GCA CTT CTT TTT - 3' (XIVb)

may advantageously be used, e.g. together with a general primer or a second specific primer. Preferably at least one of the primers of formula Va to IXb is used, particularly preferably at least one of the primer pairs Va and Vb, VIa and VIb, VIIa and VIIb, VIIIa and VIIIb, and IXa and IXb is used. Preferably at least the primer pairs Va and Vb, VIa and VIb and VIIa and VIIb are used; more preferably all five of the primer pairs Va to IXb are used as several pathogenic Pythium species are responsible for cavity spot. As with the primers of formulae Ia to IVb, these can be prepared by conventional synthetic methods, in particular solid state synthesis.

The amplified DNA sequences produced using primer pairs Va and Vb, VIa and VIb, VIIa and VIIb, VIIIa and VIIIb and IXa and IXb are respectively about 646, 352, 380, 330 and 329 bp.

The primer pairs used in the assay method of the invention clearly should not hybridize to the DNA of the vegetable (e.g. carrot) itself.

While the method of the invention is particularly suited for use on carrots and soil from fields in which carrots are to be grown or are growing, it is also more generally applicable to fields for vegetable (in particular root vegetable) and potato production, e.g. parsnip, celery, lettuce, potato and brassica.

The invention will now be illustrated further by the following non-limiting Examples.

Examples 1 to 4

Primers of formulae IIIa to IVb

These were ordered by formula and prepared commercially by Eurogentec, Serang, Belgium using conventional methods. Alternatively these may be prepared on a

support matrix using a Pharmacia Gene Assembler Plus instrument. The primers produced are then deprotected and cleaned from the support matrix by overnight incubation at 55°C in 1 mL ammonia. Blocking groups and ammonia may be removed by chromatography on a Pharmacia NAP 10 column with the primer being eluted in 1 mL water. Primer concentration can then be estimated spectrophotometrically using the factor 1 AU = 20 $\mu g\ mL^{-1}$ at 260 nm.

Example 5

DNA extraction from soil

A FastDNA SPIN kit for Soil (available from Qbiogene Inc/ Bio 101 is used in this Example. A soil sample is collected and treated as follows:

1. Add 300 - 500 mg of soil to Multimix Tissue matrix Tube and place on ice. Process in FastPrep instrument for 20 seconds at speed 4.5 and place on ice. Add 980 μl Sodium Phosphate Buffer and 122 μl MT Buffer and process in FastPrep instrument for 30 seconds at speed 5.5 and place on ice
2. Centrifuge at 14,000 xg for 15 minutes and place on ice
3. Transfer supernatant to new tubes (1.5 ml tubes) and add 250 μl PPS
4. Mix by inverting the tubes by hand 10 times and centrifuge at 14,000 xg for 5 minutes
5. Transfer supernatant to new tubes (2 ml tubes), add 1 ml RESUSPENDED Binding Matrix Suspension and invert by hand for 2 minutes
6. Centrifuge at 14,000 xg for 5 seconds and discharge supernatant
7. Resuspend in 1 ml of 5.5M Guanidine Thiocyanate
8. Centrifuge at 14,000 xg for 5 seconds and discharge supernatant
9. Resuspend in 600 μl of 5.5M Guanidine Thiocyanate

and transfer to new tubes with Spin Filters

10. Centrifuge at 14,000 xg for 1 minute and empty catch tube
11. Add 500 µl SEWS-M to the Spin Filter (Wash 1) and resuspend matrix
12. Centrifuge at 14,000 xg for 1 minute and empty catch tube
13. Add 500 µl SEWS-M (aqueous salt/ethanol solution) to the Spin Filter (Wash 2) and resuspend matrix
14. Centrifuge at 14,000 xg for 1 minute and empty catch tube
15. Centrifuge 14,000 xg for 2 minutes
16. Place Spin Filters in new catch tubes and air dry for 5 minutes
17. Add 100 µl DES and resuspend matrix
18. Centrifuge at 14,000 xg for 1 minutes
19. Store in fridge or at -20°C.

Example 6

DNA extraction from carrot peel

A GenElute Plant Genomic DNA kit (available from Sigma, St. Louis, Missouri, USA) is used in this Example. The carrot tissue sample is prepared by rinsing the carrot in water then peeling one third of the length of the top and tip. The peel is freeze dried then ground to powder. DNA extraction then proceeds as follows:

1. Place about 50mg dried carrot tissue powder in a microfuge tube
2. Add 700 µl of Lysis Solution Part A and 100 µl of Lysis Solution Part B
3. Mix by vortexing and inversion and incubate at 65°C for 10 minutes with occasional inversions
4. Add 260 µl Precipitation Solution and mix by inversions
5. Place on ice for 5 minutes

6. Centrifuge at 14,000 xg for 5 minutes (to pelletize cellular debris, proteins and polysaccharides)
7. Carefully transfer supernatant to a filtration column (BLUE filter in a collection tube)
8. Centrifuge at 14,000 xg for 1 minute and discard the filtration column
9. Add 700 µl of Binding Solution and mix by pipetting up and down 3 times
10. Transfer about 700 µl to a Nucleic Acid binding column (COLORLESS insert with a RED O-RING in a collection tube)
11. Centrifuge at 14,000 xg for 1 minute and empty the collection tube
12. Transfer the remainder of the liquid from step (9) to the Nucleic Acid binding column
13. Centrifuge at 14,000 xg for 1 minute and discard the collection tube
14. Place column in a new collection tube and add 500 µl diluted Washing Solution (Wash 1)
15. Centrifuge at 14,000 xg for 1 minute and empty collection tube
16. Add 500 µl diluted Washing Solution (Wash 2)
17. Centrifuge at 14,000 xg for 1 minute
18. Transfer column to new collection tube and air dry for 5 minutes
19. Elute DNA with 100 µl pre-warmed (65°C) Elution solution by centrifugation at 14,000 xg for 1 minute.

Example 7

DNA Purification

For this Example, Micro Bio-Spin Chromatography columns (available from BioRad) and insoluble polyvinylpolypyrrolidone powder (P6755 from Sigma) are used. DNA purification is then effected as follows:

1. Place column in a 1.5 ml centrifuge tube

2. Fill column with polyvinylpolypyrrolidone powder to 1 mm below the edge and add 400 ml double distilled H_2O
3. Centrifuge at 4,000 rpm (tabletop centrifuge) for 5 minutes
4. Transfer column to new 1.5 ml centrifuge tube and add DNA extract from Example 5 or 6
5. Centrifuge at 4,000 rpm (tabletop centrifuge) for 4 minutes and discharge column
6. Store DNA at -20°C.

Examples 8 and 9

DNA amplification

The reactions are done in a total volume of 25 µl and the PCR reaction mixture is prepared as follows:

13.75 µl	H_2O
2.5 µl	10 x PCR buffer containing 15 mM $MgCl_2$ (Roche)
2.5 µl	dNTP 2 mM
2.5 µl	BSA (bovine serum albumin) 1 mg/ml
1.25 µl	Forward primer (20 pmol/µl)
1.25 µl	Reverse primer (20 pmol/µl)
0.25 µl	Taq DNA polymerase (Roche) 5U/µl
1.0 µl	DNA template

The PCR program used is:

1.	Denaturation	94°C 5 min
2.	45 cycles of	94°C 20 sec, 62°C 30 sec, 72°C 30 sec
3.	Terminal elongation	72°C 2 min
4.	Storage	4°C

After amplification, 10 µl of the PCR product are added to 2 µl DNA loading buffer and run on a 1.2% agarose gel in 1 X TBE or 1 X TAE buffer at 100V for 45 minutes.

In Example 8, the forward and reverse primers are the primers of formulae IIIa and IIIb of Examples 1 and 2. In Example 9, the forward and reverse primers are the primers of formulae IVa and IVb of Examples 3 and 4.

Example 9

Sensitivity

The primer pairs of Examples 1/2 and 3/4 were tested against DNA extracted from Pythium sylvatium, Pythium violae L, Pythium violae/Pythium pareocandrum like, Pythium irregulare, Pythium ultimum, Phytophthora infestans, Phytophthora megasperma, Stemphyllium sp., Verticillium sp., Fusarium "powdery poae", Fusarium sporotrichioides, Fusarium avenaceum, Fusarium sp., Microdoccium nivale, Rhizoctonia sp., Rhizoctonia solani, Cylindrocarpon sp., Botrytis sp, healthy carrot, M. acerina and F. carotea.

The results are set out in Table 1 below.

Table 1

Species	Example 1/2	Example 3/4
M. acerina	+	-
F. carotea	-	+
Healthy carrot	-	-
Pythium sylvatium	-	-
Pythium violae L	-	-
Pythium violae/ Pythium pareocandrum like	-	-
Pythium irregulare	-	-
Pythium ultimum	-	-
Phytophthora infestans	-	-
Phytophthora megasperma	-	-

Stemphyllium sp.	-	-
Verticillium sp.	-	-
Fusarium "powdery poae"	-	-
Fusarium sporotrichioides	-	-
Fusarium avenaceum	-	-
Fusarium sp.	-	-
Microdoccium nivale	-	-
Rhizoctonia sp.	-	-
Rhizoctonia solani	-	-
Cylindrocarpon sp.	-	-
Botrytis sp.	-	-

- = no DNA amplification

+ = DNA amplification

Claims:

1. An assay method for detecting fungal infection of soil or vegetables by pathogenic fungal species, said method comprising:
obtaining a sample of soil or vegetable; treating said sample to lyse fungal cells therein; using an oligonucleotide primer pair, effecting a polymerase chain reaction on DNA released by lysis of the fungal cells; and detecting DNA fragments generated by said polymerase chain reaction;
wherein said primer pair comprises an 18- to 24-mer having the ability to hybridize to one of the oligonucleotide sequences of formulae Ia, Ib, IIa and IIb, IIIa, IIIb, IVa and IVb:

5' - GTT TGA ATG GAG TCC GAC CG - 3' (Ia)
5' - CGG CGT ACT TGC TTC GGA GC - 3' (Ib)
5' - TGG GAT TAA CGG GCA GAG AC - 3' (IIa)
5' - TTT CGC ATT CGG AGG CTT GG - 3' (IIb)
5' - CGG TCG GAC TCC ATT CAA AC - 3' (IIIa)
5' - GCT CCG AAG CAA GTA CGC CG - 3' (IIIb)
5' - GTC TCT GCC CGT TAA TCC CA - 3' (IVa)
5' - CCA AGC CTC CGA ATG CGA AA - 3' (IVb).

2. A method as claimed in claim 1 wherein said primer pair comprises a pair of 18- to 24-mers having the ability to hybridize to a pair of the oligonucleotide sequences of formulae Ia and Ib or IIa and IIb.

3. An 18- to 24-mer oligonucleotide primer hybridizable to an oligonucleotide sequence selected from those of formulae Ia, Ib, IIa, IIb, IIIa, IIIb, IVa and IVb.

4. A primer composition comprising a pair of 18- to 24-mer oligonucleotide primers at least one of which is

hybridizable to an oligonucleotide sequence of formula Ia, Ib, IIa, IIb, IIIa, IIIb, IVa or IVb, optionally together with a carrier.

5. A kit for the performance of the assay method of the invention, said kit comprising at least one primer pair as defined in claim 1 together with instructions for the performance of the assay method of claim 1.

6. A process for the extraction of pathogen DNA from host vegetable tissue, which process comprises:

i) contact at least 20 mg of dry powdered plant tissue (preferably surface tissue such as peel) with at least 5 μL/mg dry tissue of an aqueous fungal cell lysing agent;
ii) incubate;
iii) mix with at least 4.5 μL/mg dry tissue of an aqueous solution of a protein and polysaccharide precipitating agent;
iv) centrifuge and collect DNA-containing supernatant;
v) filter;
vi) contact DNA-containing filtrate with a DNA-binding substrate and centrifuge;
vii) wash the DNA-carrying substrate with an aqueous ethanolic solution, centrifuge and remove the liquid phase;
viii) repeat step (vii) at least once;
ix) dry the DNA-carrying substrate; and
x) contact the substrate with an aqueous solution of a DNA release agent, centrifuge and collect the DNA-containing supernatant.

7. A kit for pathogen DNA extraction from host vegetable tissue, which kit comprises:

a) a fungal cell lysing agent;

b) an aqueous solution of a protein and polysaccharide precipitating solution;

c) a DNA-binding substrate;

d) an aqueous ethanolic wash solution; and

e) an aqueous solution of a DNA release agent;

together with instructions for the use of said kit for pathogen DNA extraction from host vegetable tissue.